FINAL REPORT ON

Evaluation of Chinook salmon otoliths to determine hatchery or natural origin in the Mokelumne River watershed in the California Central Valley:

An integrated microchemistry/microstructure approach

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Executive summary

The US Fish and Wildlife Service, Anadromous Fish Restoration Program (AFRP) requires reliable estimates of the relative abundance of hatchery and naturally produced Chinook salmon (CS) returning to California's Central Valley (CCV) to effectively plan and evaluate restoration measures. Here, we combine two new otolith-based methods—microchemical analysis and microstructural analysis—to estimate the relative abundance of hatchery and naturally produced adult CS in the 2004 escapement to the Mokelumne River watershed. This study is the first application of these methods to quantify the population structure of adult CS spawners. By combining the two methods, we provide a robust estimate of relative abundance in the sampled population and also provide performance data for both methods, which can be used in planning future studies.

The study included method validation and comparison and two phases of analysis. In Phase 1, we determined the relative abundance of hatchery and natural origin adult CS in the 2004 escapement, and in Phase 2, we compared the relative abundance of hatchery and natural origin adult CS spawning in the river versus in the hatchery.

Method validation was integrated into the study. Otoliths from thirteen coded wire tag (CWT) adult CS from the Mokelumne River Hatchery were analyzed blindly by both methods as part of the first phase of the study. Microchemistry correctly identified all of the CWT fish as hatchery origin (13 of 13; 100% correct), and microstructure correctly identified 12 of 13 (92% correct). In addition, during the course of the study 25 independent analyses were made for 21 CS of known origin, all of which were correctly identified by microchemistry. The misclassification rate for microstructural analysis in this study is consistent with previous work showing a 10% misclassification rate based on the feeding ecology of hatchery and natural origin fish (Barnett-Johnson et al. 2007). This rate of misclassification is used to estimate the adult population structure with the microstructure data. The microchemistry method error rate is low and therefore those data are used directly to estimate the adult population structure.

For Phase 1, we used both methods to independently evaluate otoliths from 100 adult CS. Both methods estimated that the relative abundance of natural origin CS returning to the Mokelumne River watershed in 2004 was 3% (1-8% 95% confidence interval). This estimate is in agreement with an estimate made of 3% from coded wire tag (CWT) adult CS data for the same year (EBMUD unpub. data). These estimates are within error of the relative production of hatchery and natural juvenile production for the Mokelumne River watershed in 2000 through 2002, the primary years contributing to the 2004 escapement. Based on these data, the returning adult population structure reflects juvenile production, and survival for naturally produced juvenile CS is between ½ and 4 times the survival rate for hatchery juvenile CS from same period.

For Phase 2, we evaluated an additional ~100 adult salmon otoliths by both methods to achieve a total of ~100 classifications for both the hatchery and in-river escapement groups. Based on microchemistry, the in-river spawning group had statistically more natural origin CS than the hatchery (10% vs. 3%; 95% CI: 5-18% vs. 1-8%; $p<0.05$). These data support the hypothesis that adult CS can home to the location of spawning within a watershed, which has previously been shown in other watersheds (Hendry et al. 2000).

We estimate that microstructural analysis is approximately one-third the cost of microchemical analysis per otolith (~$160/otolith versus ~$425/otolith). However, considering

the lower error rate of microchemistry, the cost per given confidence interval is approximately the same. Economies of scale would significantly reduce the costs for both methods, but the potential for cost reduction is greater for microstructure (~1/2) because the major cost is labor, whereas for microchemistry the cost reduction would be less (~1/3) because of the cost of microchemical analysis (~$100/otolith).

Objectives: Accomplished as proposed

- Combined the otolith microchemistry and otolith microstructure methods to distinguish hatchery and naturally reared CS to provide optimal data on population structure.
- Applied these new otolith-based methods for distinguishing hatchery and naturally reared CS to the otolith sample set collected for the Mokelumne River watershed in the Fall of 2004.
- Determined the relative abundance of hatchery and naturally reared adult CS that returned to the Mokelumne River watershed in the fall of 2004.
- Determined the relative abundance of hatchery and naturally reared CS in both the Mokelumne River in-river spawning population and the hatchery recovered population in the fall of 2004.
- Provided information on the cost, accuracy, and precision of the two methods, and identify optimal solutions for specific management scenarios important to the AFRP for distinguishing hatchery and naturally reared CS in the CCV.
- Provided age estimates to determine age structure of spawners for the Mokelumne River watershed in the fall of 2004.

Background

The role of artificial propagation in recovering threatened and endangered populations to sustainable levels is one of the most controversial issues in applied ecology. A central issue for fisheries ecology is whether hatcheries are effective or whether they may actually harm natural populations (Myers et al. 2004). Impacts of hatchery-produced salmon on the long-term sustainability and persistence of naturally spawned salmon have been difficult to assess ((NRC) 1996). In the CCV, traditional tagging methods (e.g., physical or genetic) are not sufficient to independently quantify the status of wild populations (Banks et al. 2000). Population estimates of wild salmon are inferred from survivorship estimates of CWT marked hatchery fish recovered in commercial and recreational fisheries or when adults return to hatcheries to spawn. Due to the historically small proportion of CWT marked fish (<10% hatchery releases, and in some cases as low as 0%), population estimates have relied on large expansions from few returned tags introducing substantial error with an underlying assumption of equal survival rates for wild and hatchery fish.[1] Parameterizing values for survivorship in stock assessment models has been difficult because survival rates for hatchery fish have been shown to differ from wild fish with conflicting evidence as to the predicted directionality of effect (Lichatocwich 1999; Levin et al. 2001). If it were possible to distinguish all hatchery from wild CS, it would be possible to (i) determine estuary and marine survival trends for hatchery and wild fish, (ii) track the decline or recovery of wild populations independently of hatchery supplementation, (iii) detect straying of hatchery fish to breeding grounds of wild salmon populations, (iv) quantify potential

[1] Final report on anadromous salmonids fish hatcheries in California. California Department of Fish and Game & National Marine Fisheries Service Southwest Region Joint Hatchery Review Committee. December 3, 2001. Copies available at the National Marine Fisheries Service, Santa Cruz Laboratory library 110 Shaffer Rd. Santa Cruz, CA 95060.

demographic impacts of hatcheries, and (v) evaluate the success of restoration plans in increasing natural production.

To specifically meet the need to determine the relative abundance of hatchery and naturally reared salmon in the CCV, new otolith-based methods have been developed to identify hatchery and naturally produced salmon using these naturally produced tags. Otoliths are calcium carbonate structures in the inner ear of bony fish that accrete with daily and seasonal growth increments, preserved by successive growth layers (Neilson & Geen 1982; Campana & Neilson 1985). Weber et al. (2002) developed a method based on an isotopic signature recorded in the otolith (microchemistry). Barnett-Johnson et al. (2007) developed methods based on otolith growth patterns (microstructure), which they used to estimate the relative contribution of naturally produced adult CS to the fishery off the central California coast. Their results suggest that 10% of the fishery is naturally produced. It should be noted that these fish could be the progeny of hatchery parentage, and therefore in this study, we use the term "naturally produced", not "wild", for fish that hatched from an egg in a river.

The next step in assessing the sustainability of the natural population is to determine the stock structure on spawning grounds. The Mokelumne River watershed provides an ideal system to evaluate the utility of both techniques in determining stock composition and to identify and resolve potential scaling issues—including method cost, accuracy, and precision—before committing resources to a CCV-scale study.

Here we combine the microchemistry and microstructural methods to characterize the population structure in the 2004 escapement to the Mokelumne River watershed. By combining the two methods, this study provides answers to important questions surrounding hatchery and natural population composition of Mokelumne River CS and also provides a sound foundation from which to make decisions about what approach to take for future studies.

Methods

Sample. Otoliths were collected from 363 adult CS the Mokelumne River during spawning carcass surveys and ladder trapping of adult CS at Woodbridge Irrigation District Dam (WIDD) conducted by biologists for the East Bay Municipal Utility District between October 2004 and January 2005[2] (Table 1). Otoliths were collected from 947 CS during spawning at the Mokelumne River Hatchery during the same period. Otoliths were also collected from 15 CWT CS, which were determined to be from the Mokelumne River Hatchery based on CWT analysis completed at the California Department of Fish and Game (CDFG) head lab in Santa Rosa and submitted to the Regional Mark Information System database (RMIS). The fish ladder providing entrance into the Mokelumne River Fish Hatchery was operated from October 12, 2004 through April 30, 2005 (Anderson 2005)[3]. Otoliths were extracted in the field, rinsed, and stored dry until mounting. All otoliths were provided to the USFWS, where they were assigned random numbers

[2] **Workman, M.L. 2005. Lower Mokelumne River Upstream Fish Migration Monitoring Conducted at Woodbridge Irrigation District Dam. August 2004 through July 2005. Report to East Bay Municipal Utility District.**

[3] **Anderson, R.A. 2005. Annual Report Mokelumne River Fish Hatchery. California Department of Fish and Game report.**

and supplied for preparation and analysis without any supporting information, including location of collection.

The work was conducted in two phases. In phase 1, we determined the relative contribution of natural and hatchery production to the Mokelumne River watershed as a whole and compared the two methods. The proportion of in-river spawners to hatchery spawners represented in the otolith sample is double the in-river to hatchery proportion in the escapement estimate. To eliminate this sampling bias towards in-river spawners, a random sample was taken independently from the two habitats to create a subsample that proportionally represents the escapement estimates. To achieve this goal, otoliths from 87 CS were collected at the hatchery and 13 CS collected in-river. This proportion was determined based on the estimate of the relative escapement to the river and the hatchery (Table 1). Thirteen CWT CS were mixed in with these samples to test the accuracy of the assignments using the otolith-based methods.

In Phase 2, we tested the hypothesis that the two spawning locations (e.g., in-river and hatchery) have different relative proportions of hatchery and naturally produced fish. To achieve these goals, we evaluated otoliths from an additional 13 CS collected in the hatchery and 87 collected in-river. Using samples from both project phases achieved a balanced sampling design of 100 classifications for the hatchery and 100 for in-river escapement groups to test this hypothesis.

In addition to the 2004 adult CS samples, otoliths from hatchery and naturally produced juvenile CS from the Mokelumne River were included in the study to determine the difference in sulfur isotopic composition between these habitats. Also, known naturally produced Spring-run adult CWT CS from Butte Creek, 1 naturally produced Winter-run CS from the Sacramento River, 1 naturally produced juvenile CS from the Salmon River system in Idaho, and 1 known hatchery adult CWT CS from the Feather River Hatchery were included in the study to test accuracy of the microchemistry technique. The mounts for these samples varied in quality. The 2007 Butte Creek samples were relatively roughly prepared and previously analyzed by laser ablation.

Table 1. Mokelumne River watershed adult Chinook salmon escapement and sampling in 2004. The proportion of in-river spawners in the sample is double the estimated in-river escapement. To eliminate this sampling bias towards in-river spawners, a random sample was taken independently from the two sample groups to create a subsample that proportionally represents the escapement estimates.

	2004 fall run Chinook salmon escapement	percent	Fish sampled for otoliths	percent	Sample as percent of escapement	Proportional sample
Hatchery	10,356	87%	946	72%	9%	87
In-river	1,588	13%	363	28%	24%	13
Total	11,944	100%	1,309	100%	11%	100

Otoliths were first aged whole with transmitted light. Then, otoliths were embedded in epoxy and polished on both sides to reveal internal structure necessary for microstructural and microchemical analysis of the juvenile growth portion of the otolith. Otoliths were polished so that the daily growth bands after the exogenous feeding check were clearly visible, criteria

particularly important for microstructural analyses. Otoliths were transferred and mounted individually on 1 inch diameter glass rounds, and the otolith surface was polished flush with the surface of the surrounding epoxy, such that the otolith core was exposed. Achieving a flat polished surface is imperative for accurate microchemical analyses. Since both microstructural and microchemical analyses were conducted on the same sample, some additional effort beyond preparation for either individual method was required.

Microstructural analysis. Microstructural analysis was conducted using the methods detailed in Barnett-Johnson et al. (2007). Briefly, otolith sections were observed using a compound microscope (200x) under transmitted polarized light, imaged with a digital camera (Nikon DXM1200®), and analyzed with the aid of image analysis software (Optimas® v 6.5) and a customized otolith macro program. Widths of 30 daily increments beginning at the exogenous feeding check and progressing toward the margin were measured (Fig. *). Variability in widths was characterized using the coefficient of variation (CV). The prominence of the exogenous feeding check was categorized and scored as not distinct (1), intermediate (1.5), or distinct (2) based on whether it was visible along the entire circumference and isolated from other surrounding dark bands.

Microchemistry. Otolith microchemistry analysis was performed using the method of Weber et al. (2002). The otoliths were analyzed in two sessions by secondary ion mass spectrometry (SIMS) at the UCLA Keck Center for Geological SIMS using an ims1270 (Cameca, Genevilliers, France). For both sessions, the primary ion current was ~1.5 pA Cs^+ with Kohler illumination producing a ~30 micron analysis spot on the sample. The secondary ions $^{32}S^-$ and $^{34}S^-$ were collected primarily in peak hopping mode for the first session and static collection mode for the second session. To determine the origin (hatchery or natural) of each individual fish, the otolith growth region immediately beyond the exogenous feeding check (post exogenous feeding, or PEF)) was targeted for $\delta^{34}S$ analysis. For otoliths in which the exogenous feeding check was indistinct, analyses were made between 250 microns 400 microns to increase the probability of detecting variations in otolith $\delta^{34}S$. These PEF analyses were compared to $\delta^{34}S$ analyses of the otolith region before exogenous feeding (core) or after marine entry. This internal standardization method increases measurement precision because differences between otolith mounts can result in shifts in instrumental mass fractionation (IMF). For one session, the variation in the $\delta^{34}S$ analyses of the marine portion of all analyzed otoliths exceeded the precision of the individual measurements by ~50% (2SD = 5.7 ‰ vs. 2SE ~4 ‰, respectively; SD=standard deviation; SE=standard error). In some cases, internal referencing was not used because the exposed plane of the otolith was potentially above the otolith core. In these cases, the data are referenced to the average marine $\delta^{34}S$ value found in the marine growth region of other otoliths. Uncertainty in the average marine $\delta^{34}S$ value is based on the overall variability in these measurements.

Data reduction. The absolute sulfur isotopic composition of the otolith has not been established. For the purposes of sample classification, relative values can be used. As part of this study, we

estimate the isotopic composition of the core and marine regions of growth, and use those estimates to standardize the PEF. Uncorrected $^{34}S/^{32}S$ ratios are converted to $\delta^{34}S$ values in parts permil (‰) relative to the Canyon Diablo Troilite (CDT) $^{34}S/^{32}S$ standard ratio (0.0450045) using:

$$\delta^{34}S_{raw} = [(^{34}S/^{32}S)_{raw} / (^{34}S/^{32}S)_{CDT} - 1] * 1000‰. \quad \text{(eq. 1)}$$

Note that $\delta^{34}S_{raw}$ is not corrected for IMF and therefore is not standardized to CDT. When possible, PEF $\delta^{34}S$ data are corrected for IMF based on internal reference analyses:

$$\delta^{34}S_{PEF\text{-}est} = \delta^{34}S_{Ref\text{-}est} + (\delta^{34}S_{Ref\text{-}raw} - \delta^{34}S_{PEF\text{-}raw}), \quad \text{(eq. 2)}$$

where $\delta^{34}S_{PEF\text{-}est}$ is the estimated PEF $\delta^{34}S$ value relative to CDT, $\delta^{34}S_{Ref\text{-}est}$ is the estimated $\delta^{34}S$ value for the reference region relative to CDT, and $\delta^{34}S_{Ref\text{-}raw}$ and $\delta^{34}S_{PEF\text{-}raw}$ are the mean uncorrected $\delta^{34}S$ values for the reference and the PEF regions, respectively. Standard error propagation is performed by summing the standard errors (SE) for the mean $\delta^{34}S$ values for PEF, internal reference, and mean of the reference region in quadrature. This error estimate is for total internal error across samples and does not take into account the uncertainty in the absolute CDT $\delta^{34}S$ value of the reference regions, which is discussed below. For PEF $\delta^{34}S$ measurements without internal reference analyses, the data are corrected for the mean IMF estimated from the marine measurements:

$$\delta^{34}S_{PEF\text{-}cor} = IMF + \delta^{34}S_{PEF\text{-}meas}, \quad \text{(eq. 3)}$$

where IMF = $\delta^{34}S_{Ref\text{-}est} - \delta^{34}S_{Ref\text{-}meas}$. For these PEF $\delta^{34}S$ estimates, internal precision is estimated by summing in quadrature the standard error (SE) for the mean PEF $\delta^{34}S$ value and the estimate of sample to sample measurement precision (SD of all marine measurements for the session).

The marine growth region is assumed to have $\delta^{34}S_{CDT}$ = 18‰, the isotopic composition of organic matter in the ocean (Thode 1991). In this study, we found that the marine region differs in isotopic composition from the core by 4.6±1.1‰ (mean±2 standard errors). These data are used to correct the PEF data relative to CDT.

Statistical analysis.

Watershed estimate- phase I

The rearing origins of CS adults spawning in the Mokelumne River were determined using microstructure and microchemisty methods independently. Assignments using the otolith microstructure technique used the microstructure variables (mean and variation in increment widths and prominence of exogenous feeding check) and the combined year discriminant function model previously developed from juveniles collected from wild and hatchery sources in 1999 and 2002 (Barnett-Johnson et al. 2007). However, to estimate the composition of spawners to the Mokelumne River, we used a maximum likelihood estimation procedure (HISEA) based

on finite-mixture distributions rather than deriving the composition by directly classifying individuals with the combined year DF model (Millar 1987; Millar 1990; Koljonen et al. 2005). The HISEA mixture model (Millar 1990) is more appropriate for this application because unlike DFA, it does not require prior estimates of stock composition. Similar statistical methods have been used to understand stock structure and composition for fisheries applications and assume the sampled population is representative of the fishery (Pella & Robertson 1979; DeVries et al. 2002; Fabrizio 2005). Standard errors on the estimated proportions of hatchery and natural fish were obtained through bootstrapping with 500 replicates (Millar 1990). This term includes error associated with the classification algorithm and estimates the population composition of the spawning population from our sample data.

Individuals were also assigned to hatchery or natural origin using the microchemistry method. Classification was based on an empirically determined cut off of $\delta^{34}S$ = 7‰. The proportion of fish originating from hatcheries was therefore the number identified as hatchery fish divided by the total number of fish in phase 1 (N=100). The 95% confidence interval (CI) for the microchemistry data is determined using bionomial statistics.

Habitat association- Phase II

We tested the hypothesis that the two spawning locations (e.g., in-river and hatchery) have different relative proportions of hatchery and naturally produced fish and whether the two methods gave similar findings. To assess habitat associations between rearing types, a two factor Chi-square test was conducted for each assignment method and results were compared.

Method comparison

To test whether the two methods yielded the same results, we compared them in three ways. First, the proportions of fish assigned to hatchery or natural origin were compared using a two factor Chi square test (SYSTAT ® v 10.2). The Cohen's kappa was calculated to determine the extent to which there was agreement on individual assignments between the two methods. Values of kappa greater than 0.75 indicate strong agreement beyond chance, values between 0.40 and 0.79 indicate fair to good, and values below 0.40 indicate poor agreement. Lastly, known hatchery origin fish (the 13 CWT fish) were used to determine the accuracy of each method.

Results and Discussion

Method validation and comparison

Otoliths were analyzed from CWT adult CS and known origin juvenile CS for method validation. Of the 15 sets of otoliths collected for Mokelumne River Hatchery CWT CS, 13 were analyzed, one was overpolished, and one was misformed and was not analyzed. These were analyzed blindly as part of Phase 1 for microchemistry and microstructure. Microchemistry correctly identified all of the CWT fish as hatchery origin (13 of 13; 100% correct), and microstructure correctly identified 12 of 13 (92% correct; Table 2). In addition, 25 independent analyses were made for 21 CS of known origin, all of which were correctly identified by microchemistry (Table 3).

The results for microchemical analyses are consistent with expectations. The microchemistry method is based on the known difference between the sulfur isotopic composition of the hatchery diet, which is based on marine fishmeal, and the freshwater prey items (Weber et al. 2002). Therefore, the accuracy of identification is based on the accuracy of the microchemical analysis. Accuracy was maintained by analysis of known samples, internal standardization, and rerunning samples for which the initial analysis had low classification confidence, as well as random reruns. For the unknowns, a total of 29 out of 205 samples were rerun once, and two samples were rerun twice. The data for Phase 1 show good separation (Fig. 1). Based on these data, we conclude that our approach to the microchemistry method has greater than 95% accurate. We do not have sufficient data to determine if accuracy is higher than 99%.

The results for the microstructural analyses are also consistent with expectations. This method is based on differences in otolith microstructural development in hatcheries as compared to in the wild and has previously undergone validation (Barnett-Johnson et al. 2007). They observed a natural error rate of 10% based on differences in the feeding ecology of individual juvenile CS.

Table 2. Method Validation 1: Mokelumne River adult CWT Chinook salmon microchemistry and microstructual analysis. Values in 'Hatchery' and 'Natural' columns are posterior probabilities of assignments.

Sample	Age	Microchemistry assignment	$\delta^{34}S$ (~CDT)	2SE	Microstructure assignment	Hatchery	Natural
AFRP054	3	Hatchery	12.7	4.5	Hatchery	0.998955	0.001045
AFRP077	3	Hatchery	10.4	4.8	Hatchery	0.999522	0.000478
AFRP1086	3	Hatchery	12.0	5.5	Hatchery	0.999711	0.000289
AFRP1111	3	Hatchery	14.6	5.5	Hatchery	0.999624	0.000376
AFRP1114	2	Hatchery	15.2	2.1	Hatchery	0.999596	0.000404
AFRP1205	4	Hatchery	12.1	1.7	Hatchery	0.921828	0.078172
AFRP191	3	Hatchery	17.2	1.9	Natural	0.022152	0.977848
AFRP413	2	Hatchery	13.3	1.6	Hatchery	0.999708	0.000292
AFRP514	3	Hatchery	13.5	2.1	Hatchery	0.999091	0.000909
AFRP545	3	Hatchery	15.0	2.8	Hatchery	0.999518	0.000482
AFRP661	3	Hatchery	11.4	1.8	Hatchery	0.999152	0.000848
AFRP912	3	Hatchery	14.8	5.2	Hatchery	0.999768	0.000232
AFRP928	3	Hatchery	15.8	1.4	Hatchery	0.999619	0.000381

Table 3. Method Validation 2: Other known-origin Chinook salmon microchemistry

Source	ID Number	$\delta^{34}S$ (~CDT)	2SE	Reference*	Notes
Session 1					

Mokelumne R	20182H	16.0	3.2	2	Hatchery juvenile
Mokelumne R	20170H	12.8, 15.8	0.9, 5.5	2	Hatchery juvenile
Mokelumne R	20131H	15.3, 18.5	1.8, 5.8	2	Hatchery juvenile
Butte Cr	8R	2.5, 1.8	6.4, 7.0	2,3	Natural spring run juvenile
Butte Cr	8R	1.5, 3.4	2.2, 5.7	2,3	Natural spring run juvenile; run 2
Idaho	860621	6.7	3.1	2	Natural juvenile, Idaho
Mokelumne R	20529W	0.0	5.5	3	Natural juvenile
Mokelumne R	20531W	-1.3	6.2	3	Natural juvenile
Mokelumne R	20545W	3.1	1.6	2	Natural juvenile
Mokelumne R	20545W	3.9	1.4	2	Natural juvenile; run 2
Session 2					
Mokelumne R	J20170H	14.4	3.3	2	Hatchery juvenile
Mokelumne R	J20182H	11.9	3.9	2	Hatchery juvenile
Feather R	1	12.1	1.8	1	Hatchery adult; CWT
Mokelumne R	J20529W	7.8	2.6	2	Natural juvenile
Mokelumne R	J20545W	5.3	2.1	2	Natural juvenile
Sacramento R	3	2.5, 3.5	2.2, 2.4	2,1	Natural winter run adult
Butte Cr	10	5.0, 6.3	2.5, 2.6	2,1	Natural spring run adult; CWT
Butte Cr	10	3.5, 7.1	2.6, 3.4	2,1	Natural spring run adult; CWT; run 2
Butte Cr	10	6.8	3.5	1	Natural spring run adult; CWT; run 3
Butte Cr 2007	41	5.3	1.9	1	Natural spring run adult; CWT
Butte Cr 2007	42	2.0, 4.2	2.9, 1.6	2,1	Natural spring run adult; CWT
Butte Cr 2007	44	5.8	1.4	1	Natural spring run adult; CWT
Butte Cr 2007	46	5.4, 8.3	6.3, 1.9	2,1	Natural spring run adult; CWT
Butte Cr 2007	48	6.3, 7.2	2.5, 2.3	2,1	Natural spring run adult; CWT
Butte Cr 2007	49	4.9, 4.7	3.0, 1.4	2,1	Natural spring run adult; CWT

*Reference codes: **1=marine, 2=core, 3=external; for rows with two references, one set of juvenile otolith analyses are compared to both.**

The comparison of the two methods for the unknown samples follows the validation data. Both techniques indicate that the majority of the fish are hatchery fish (94% sulfur; 91% microstructure). Out of the total 200 fish analyzed, 179 fish were identified as hatchery fish with both methods, 10 fish were identified as naturally produced fish with both methods, 8 fish assigned hatchery using sulfur were classified as natural using microstructure, and 3 fish assigned natural by sulfur were classified as hatchery using microstructure (Table S1). Therefore, 189 fish (95%) were assigned to the same origin using both methods, and 11 fish were in disagreement. These 11 fish are presumed to be misclassified by microstructure based on the validation studies (Table 4). These results are in-line with expectation as the microstructure technique was predicted to falsely detect a greater number of natural fish than hatchery fish based on a greater number of hatchery fish in the population and a 10% error rate with the technique. Nonetheless, there is strong agreement between fish classification techniques. Chi-square tests ($p<.05$) indicate that the two tests are statistically non-independent (Table S2). Cohen's kappa values indicate good agreement between the two methods compared to chance alone (Table S3).

Table 4. Fish from the Mokelumne River watershed for which the two methods were in disagreement. Values under 'Hatchery' and 'Natural' are posterior probabilities of assignments.

Sample	Microchemistry assignment	$\delta^{34}S$ (~CDT)	2SE	Microstructure assignment	Hatchery	Natural
AFRP1026	Hatchery	14.4	2.6	Natural	0.00026	0.99974
AFRP191	Hatchery	17.2	1.9	Natural	0.022152	0.977848
AFRP199	Hatchery	12.7	1.7	Natural	0.026299	0.973701
AFRP244	Hatchery	16.7	2.8	Natural	0.17158	0.82842
AFRP395	Hatchery	15.9	1.5	Natural	0.06876	0.93124
AFRP416	Hatchery	13.6	3.0	Natural	0.025048	0.974952
AFRP637	Hatchery	12.4	4.5	Natural	0.025566	0.974434
AFRP787	Hatchery	14.1	4.5	Natural	0.148813	0.851187
AFRP565	Natural	3.5*	0.9	Hatchery	0.999406	0.000594
AFRP698	Natural	3.1*	2.5	Hatchery	0.944414	0.055586
AFRP724	Natural	3.9	3.5	Hatchery	0.999574	0.000426

*Analyzed twice.

Watershed estimate- phase I

In Phase 1 of this project, we determined the relative contribution of hatchery and naturally produced CS at the watershed scale. Samples were chosen at random from the total set of otoliths, with a proportion of hatchery and in-river collected samples of 87 to 13, respectively, to reflect the total escapement to the Mokelumne River watershed (Table 1). Fifteen CWT CS otoliths were included in this sample set at random. Based on microchemical analysis of otoliths from 95 adult CS, 92 were reared in a hatchery, and 3 were naturally reared in a river (Fig. 3), yielding an estimate of 3% naturally produced adult CS in the 2004 escapement to the Mokelumne River watershed. The 95% confidence interval (CI) for this estimate is 1 to 8%.

For the 93 adult CS classified by otolith microstructural analysis, 85 are classified as hatchery origin and 7 are classified as natural origin (Fig. 4). Two otoliths were damaged after sulfur data was collected and prior to microstructure data collection. The microstructure data are resampled using a Millar HISEA model (Table S4) to account for the natural 10% misclassification rate estimated for this method. This analysis results in an estimate of 3% naturally produced adult CS in the 2004 escapement to the Mokelumne River watershed. The model estimates the 65% CI (1 standard error) for this estimate to be 0 to 6%. At this point, we are not able to assign a 95% CI based on the model results.

The estimates by the two methods are in very good agreement with each other and with CWT data generated by Michelle Workman at EBMUD (3% natural origin; Table S5). We do not have a CI estimate for this estimate. Nonetheless, the very close agreement in the mean estimates for these three orthogonal methods, suggests that the actual relative abundance of natural origin CS in the population is close to the 3 percent mean estimate reached by microchemistry and microstructure.

These estimates of the relative abundance of natural origin adult CS in the 2004 escapement are within error of estimates of natural production of juvenile CS in the Mokelumne River watershed for the three major contributing brood years, 2000, 2001 and 2002 (Fig. 5, S1). This result suggests that the survival rates are similar for natural origin and hatchery origin juvenile CS. However, given the small percentage of natural juvenile production and the uncertainty in the escapement estimates, the survival rate for the natural origin juvenile CS could be as high as four times higher than hatchery juvenile CS survival. Furthermore, this comparison does not include the uncertainties in the estimates of juvenile production, and the release location for the hatchery juvenile CS may not be comparable to the monitoring locations that the natural production numbers are based on. Nonetheless, the results of this study place constraints on the difference in survival for natural relative to hatchery origin juvenile CS at between 50 and 400%.

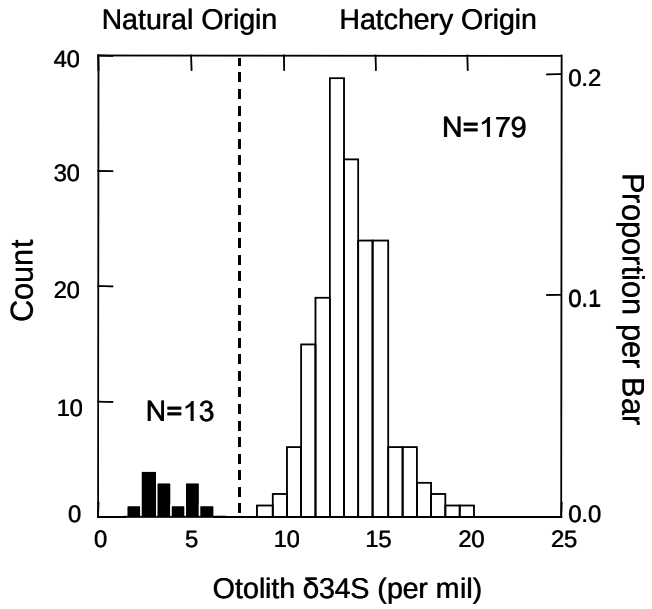

Fig. 1. [JD11] Histogram of otolith $\delta^{34}S$ for the juvenile rearing portion of the adult otolith for 192 [JD12] Chinook salmon from the 2004 escapement to the Mokelumne River watershed, Phase 1 and 2. Natural origin fish (black bars) had $\delta^{34}S$ values less than 7 and did not overlap with hatchery origin $\delta^{34}S$ values (open bars).

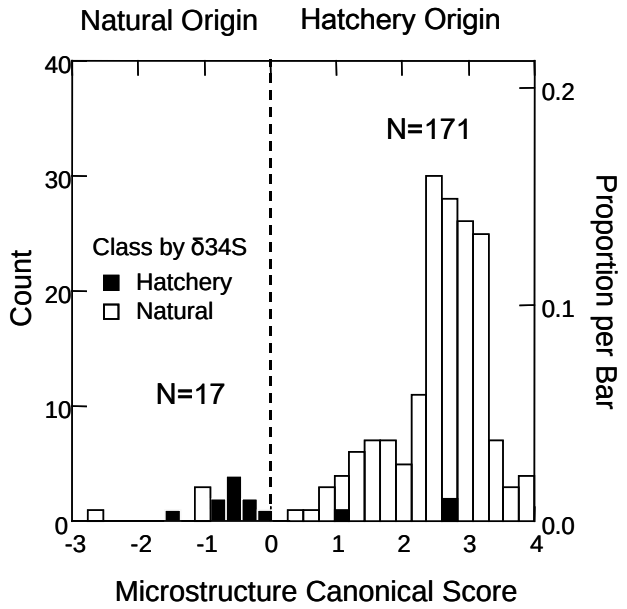

Fig. 2. Histogram of microstructure canonical score for the juvenile rearing portion of the adult otolith for 188[JD15] Chinook salmon from the 2004 escapement to the Mokelumne River watershed, Phase 1 and 2. Individuals with hatchery $\delta^{34}S$ values are represented with black bars and natural origin $\delta^{34}S$ values with open bars. Canonical value for hatchery fish (<0) and natural origin fish (separated by dashed line) determined by previous validation work (Barnett-Johnson et al. 2007)

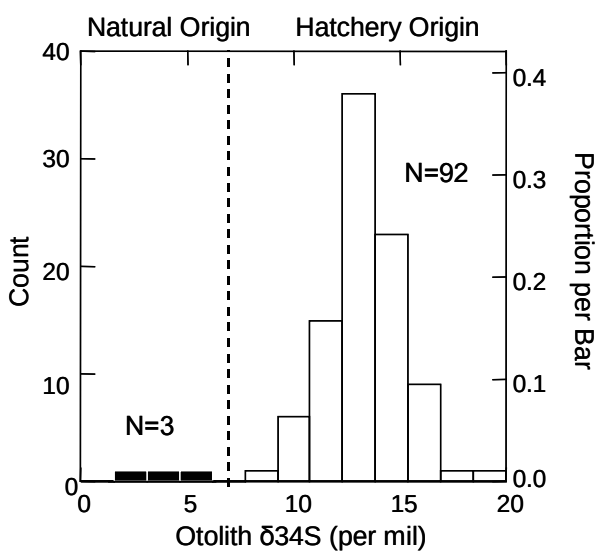

Fig. 3. Histogram of otolith $\delta^{34}S$ for the juvenile rearing portion of the adult otolith for 95[JD13] Chinook salmon from 2004 escapement to the Mokelumne River watershed, Phase 1.

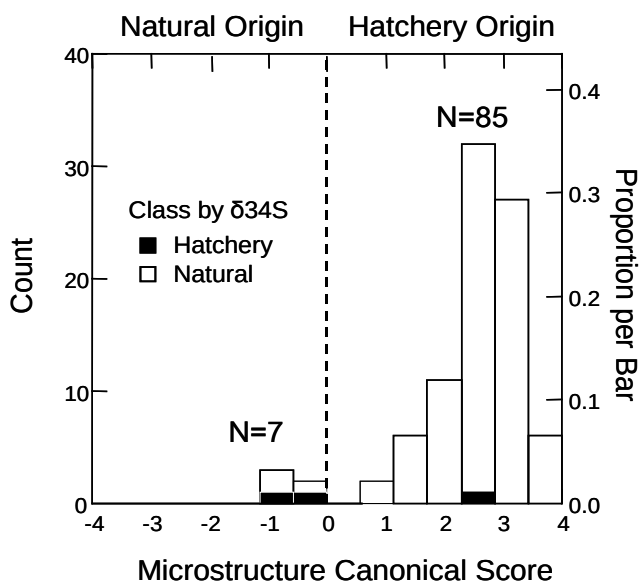

Fig. 4. Histogram of microstructure canonical score for 93[JD16] Chinook salmon from 2004 escapement to the Mokelumne River watershed, Phase 1. Individuals with hatchery $\delta^{34}S$ values are represented with black bars and natural origin $\delta^{34}S$ values (open bars).

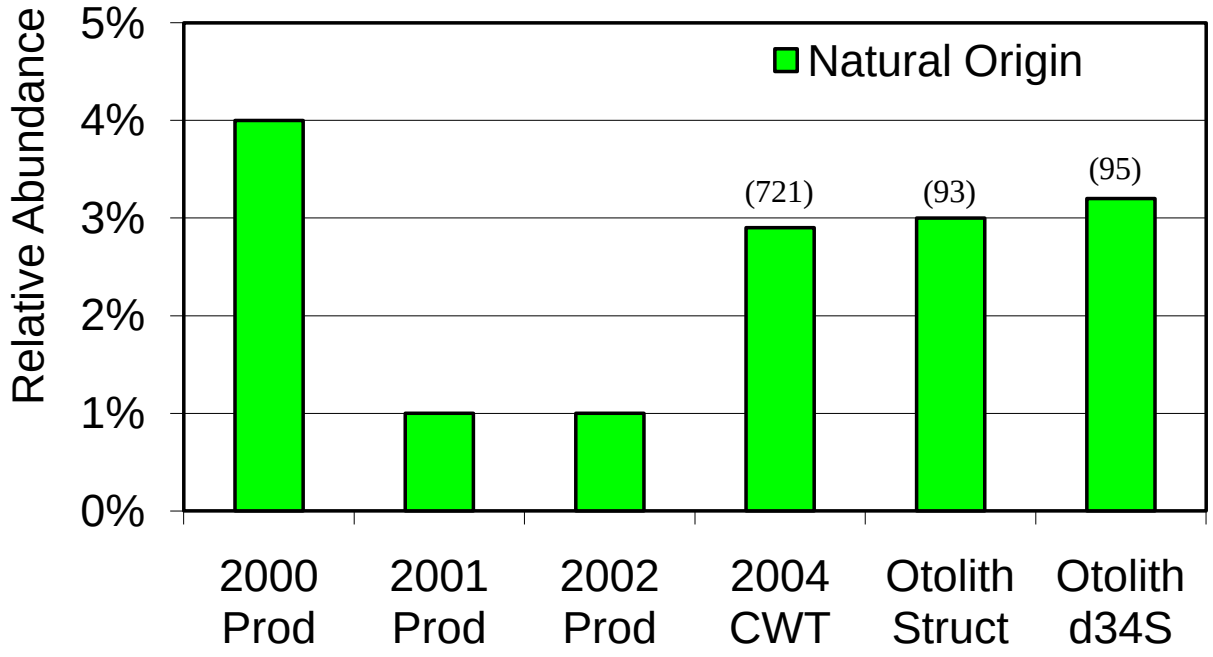

Fig. 5. Comparison of natural origin relative abundance estimates for the 2004 escapement of adult CS to the Mokelumne River watershed versus juvenile CS production in the contributing brood years.

Phase 2: Is there an in-river vs. hatchery homing tendency?

In Phase 2 of this project, we compared the relative abundance of naturally produced and hatchery produced adult CS collected in-river compared to those collected in the hatchery. A total of 95 adult CS collected in-river and 97 adult CS collected at the hatchery were assessed using microchemistry. The majority of fish spawning in the hatchery and in-river are of hatchery origin (97%). However, there is a statistically significant association of naturally produced CS spawning more frequently in-river (10 of 97, 10%; 95% CI is 4.9 to 17.6%) than in hatcheries (3 of 95, 3%; 95% CI is 1 to 8%; Table 5). Results of the Chi-square tests show that the association between production location (hatchery vs. natural) and spawning location (hatchery v. in-river) is statistically significant ($p<0.05$; Table 6). The Fisher exact test shows statistical significance at the 90% confidence level. The results using microstructure are similar (Table 7), but the results are not statistically significant (Table 8), consistent with the misclassification rate of microstructural analysis.

Table 5. Cross tabulation of microchemistry prediction against collection site.

Assigned Origin	Hatchery collection	River collection	Total

Assigned Origin	Hatchery collection	River collection	Total
Hatchery	92 (47.9%)	87 (45.3%)	179 (93.2%)
River	3 (1.6%)	10 (5.2%)	13 (6.8%)
Total	**95 (49.5%)**	**97 (50.5%)**	**192 (100%)**

Table 6. Chi-square tests of spawning location based on origin (hatchery vs. natural).

Test Statistic	Value	Degrees of freedom	p-value
Pearson Chi^2	3.888	1.000	0.049
Likelihood Ratio Chi^2	4.095	1.000	0.043
Fisher Exact Test (two-tail)			0.082

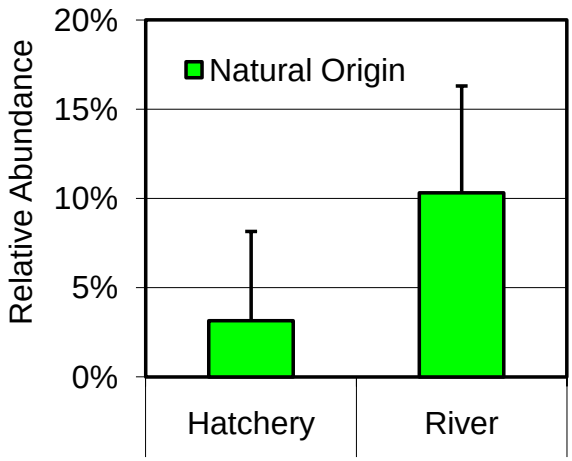

Fig. 6. Natural origin adult CS by collection site in the Mokelumne River watershed for 2004. Error bars represent 95% confidence intervals. The two estimates are statistically different at the p = 0.04 level.

Table 7. Cross tabulation of microstructure prediction against collection site.

Assigned Origin	Hatchery collection	River collection	Total
Hatchery	85 (45.2%)	86 (45.7%)	171 (91.0%)
Natural	7 (3.7%)	10 (5.3%)	17 (9.0%)
Total	**92 (48.9%)**	**96 (51.1%)**	**188 (100%)**

Table 8. Chi-square tests of microstructural data for spawning location based on origin (hatchery vs. natural).

Test Statistic	Value	Degrees of freedom	p-value
Pearson Chi^2	0.450	1.000	0.502
Likelihood Ratio Chi^2	0.453	1.000	0.501
Fisher Exact Test (two-tail)			0.614

Costs. Nominally, the cost per otolith in this study was $1000 (200+ otoliths for $210,000). However, approximately one-third of the budget went into method development, primarily for coordinating the two methods and scaling up the microchemistry method. Considering these costs, a realistic estimate for performing similar analyses at this point in time would be on the order of $600 per otolith. We estimate that microstructural analysis is approximately one-third the cost of microchemical analysis per otolith (~$200/otolith versus ~$500/otolith; Table 9). Considering the lower error rate of microchemistry, the cost per given confidence interval is approximately the same (e.g., 20% difference in cost if microstructural analysis requires data for 100 otoliths to achieve 0-10% 95% CI on 3% relative abundance, which can be achieved by microchemical analysis with data for 50 otoliths). The exact difference in cost is hard to estimate because the confidence interval for microstructural analysis is not well quantified.

Table 9. Comparison of the cost of microchemistry and microstructure otolith analysis.

	Microchemistry	**Microstructure**
This study	~$500/otolith	~$200/otolith
95% CI: 0-10%	50 otoliths $25,000	~100 otoliths ~$20,000
Instrument cost only	~$100/otolith	
Production scale	~$300/otolith	~$80/otolith

Otolith preparation methods for both techniques are compatible, but the ultimate requirements differ. Microstructural analysis requires the otolith to be polished to a very precise level to make the growth bands individually visible in the post-exogenous feeding region of the otolith. Microchemistry requires that the core and post-exogenous feeding region be exposed and smoothly polished. This difference, though seemingly small, resulted in a significant learning curve to achieve good sample preparation for both methods using the same otoliths. Preparation for only one of the two methods would have been significantly less effort.

One methodological finding of our validation work was that a simple mounting method used for microstructural analysis can be adapted for microchemical analysis. Under the standard protocol of thin section preparation for microchemical analysis by secondary ion mass spectrometry (SIMS), samples are mounted in one inch rounds that are polished flat across the entire surface. This approach minimizes instrumental mass fractionation that results from distortion of the electric field in which the secondary ions are extracted. Instrumental mass fractionation can result in erroneous isotopic measurements because it changes the measured isotopic ratio independent of sample isotopic composition. We found that otolith sections that were not in one inch rounds and were not perfectly flat could be accurately analyzed by using internal standardization. The sulfur isotopic composition of the juvenile rearing region was standardized to the otolith core and the marine growth region to correct for changes in instrumental mass

fractionation from sample mount to sample mount (Table 3, Sacramento River and 2007 Butte Creek samples). This approach to standardization was used for all samples to the extent possible. This finding demonstrates that a simple, less time-consuming and expensive sample mounting method could be used for SIMS microchemical analysis. As a result "job-shop" samples could be used for mass production, reducing the cost of sample preparation by potentially half, reducing overall cost by 10% or more.

Sample polishing, however, is still much more important for SIMS analysis than microstructural analysis. Our work showed that samples with rough surfaces (>50 micron pitting) did not run as well as samples with low surface roughness (<10 micron scratches and pits). However, samples had previously been analyzed by laser ablation ICP-MS, which leaves 40 to 100 micron holes in the sample, were successfully analyzed (Table 3, Sacramento River and all Butte Creek samples). Though not desirable, these imperfections were unimportant relative to the difference between the hatchery and natural sulfur isotopic signatures in the juvenile rearing portion of the CS otolith in the Sacramento-San Joaquin river system. It is sample roughness over a significant area of the sample that affects sample analysis, most likely through charging and distortion of the electric field.

Economies of scale would significantly reduce the costs for both methods (Table 9, S6). Labor is the biggest cost for both of these methods, and therefore, if there was sufficient work to justify training technicians, they would significantly reduce the cost. However, the potential for cost reduction is greater for microstructure because labor is essentially the only cost, whereas for microchemistry, there is a significant instrument cost (~$100/otolith).

Ultimately, when misclassification rate is taken into account, the two methods provide similar results for a similar cost. The decision regarding which method to use should therefore be guided by the following factors: number of samples available, expertise of the participants, available instrumentation, and coordination with other methods. Because of its low misclassification rate, the microchemistry method is advantageous if samples are limited and correlated analyses are being performed. A higher misclassification rate is particularly problematic for correlated analyses because the uncertainty at the individual sample level cannot be corrected for the overall misclassification rate and therefore has to be propagated to the other analyses (e.g., strontium isotopes). On the other hand, access to SIMS instrumentation can be limited, and if a large-scale study is planned, the potential for reducing costs is greater for the microstructural analysis method. In both case, a very high level of expertise is required.

Conclusion

Here we successfully determined the origin of adult CS in the Mokelumne River watershed using two methods, and we cross-validated these results with CWT data. The results show the relative proportion of hatchery and natural origin CS in the watershed reflects the relative production of hatchery and natural juvenile CS in the watershed. We also show a tendency to home to the river for spawning. This result in particular shows the benefit of the higher accuracy microchemistry method for comparing populations across space or time. However, microstructural analysis

provides data with sufficient accuracy to assess the relative abundance of hatchery and natural origin CS in a population. Both methods are viable for future studies.

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References

Banks, M.A., V.K. Rashbrook, M.J. Calavetta, C.A. Dean, D. Hedgecock (2000) Analysis of microsatellite DNA resolves genetic structure and diversity of Oncorhynchus tshawytscha in California's Central Valley. *Canadian Journal of Fisheries and Aquatic Sciences* 57, 915-927.

Barnett-Johnson, R., C.B. Grimes, C.F. Royer, C.J. Donohoe (2007) Identifying the contribution of wild and hatchery Chinook salmon (*Oncorhynchus tshawytscha*) to the ocean fishery using otolith microstructure as natural tags. *Canadian Journal of Fisheries and Aquatic Sciences* 64, 1683-1692.

Campana, S.E., J.D. Neilson (1985) Microstructure of fish otoliths. *Canadian Journal of Fisheries and Aquatic Sciences* 42, 1014-1032.

DeVries, D.A., C.B. Grimes, M.H. Prager (2002) Using otolith shape analysis to distinguish eastern Gulf of Mexico and Atlantic stocks of king mackerel. *Fisheries Research* 57, 51-62.

Fabrizio, M.C. (2005) Experimental design and sampling strategies for mixed-stock analysis. Stock Identification Methods: Applications in Fishery Science (ed. by S. X. Cadrin, K. D. Friedland and J. R. Waldman), pp. 467-498. Elsevier Academic Press, San Francisco, Calif.

Hendry, A.P., J.K. Wenburg, P. Bentzen, E.C. Volk, T.P. Quinn (2000). Isolation in the wild: Evidence from introduced salmon. Science 290:516-518.

Koljonen, M.L., J.J. Pella, M. Mesuda (2005) Classical individual assignments versus mixture modeling to estimate stock proportions in Atlantic salmon (Salmo salar) catches from DNA microsatellite data. *Canadian Journal of Fisheries and Aquatic Sciences* 62, 2143-2158.

Levin, P.S., R.W. Zabel, J.G. Williams (2001) The road to extinctions is paved with good intentions: negative association of fish hatcheries with threatened salmon. *Proceedings of the Royal Society, London B* 268, 1153-1158.

Lichatocwich, J. (1999) Salmon Without Rivers. Washington, DC: Island Press.

Millar, R.B. (1987) Maximum likelihood estimation of mixed stock fishery composition. *Canadian Journal of Fisheries and Aquatic Sciences* 44, 583-590.

Millar, R.B. (1990) A versatile computer program for mixed stock fisher composition estimation. Can. Tech. Rep. Fish. Aquatic. Sci. *1753*, 29.

Myers, R.A., S.A. Levin, R. Lande, F.C. James, W.W. Murdoch, R.T. Paine (2004) Hatcheries and endangered salmon. *Science* 303, 1980.

Neilson, J.D., G.H. Geen (1982) Otolith of chinook salmon (*Oncorhynchus tshawytscha*): daily growth increments and factors influencing their production. *Canadian Journal of Fisheries and Aquatic Sciences* 39, 1340-1347.

National Research Council (NRC) (1996) Upstream: salmon and society in the Pacific Northwest. Washington, DC: National Academy Press.

Pella, J.J., T.L. Robertson (1979) Assessment of composition of stock mixtures. *Fisheries Bulletin, USA* 77, 387-398.

Thode, H.G. (1991) Sulfur isotopes in nature and the environment: an overview. Stable Isotopes: Natural and Anthropogenic Sulphur in the Environment (ed. by H. R. Krouse and V. A. Grinenko), pp. 1-26. John Wiley & Sons, New York.

Weber, P.K., I.D. Hutcheon, K.D. McKeegan, B.L. Ingram (2002) Otolith sulfur isotope method to reconstruct salmon (*Oncorhynchus tshawytscha*) life history. *Canadian Journal of Fisheries and Aquatic Sciences* 59, 587-591.

Supplemental data

Table S1. Comparison of microchemistry and microstructure classification for all unknowns (non-CWT)

MICROSTRUCTURE_ASSIGN$(rows) by SULFUR_ASSIGN$(columns)	Sulfur Hatchery	Sulfur Natural	Total
Microstructure H	179 (89.5%)	3 (1.5%)	182 (91.0%)
Microstructure W	8 (4.0%)	10 (5.0%)	18 (9.0%)
Total	**187 (93.5%)**	**13 (6.5%)**	**200 (100%)**

Table S2. Results of Chi-square tests of association for MICROSTRUCTURE_ASSIGN$ and SULFUR_ASSIGN$

Test Statistic	Value	df	p-value
Pearson Chi-square	78.322	1.000	0.000
Likelihood Ratio Chi-square	40.890	1.000	0.000
Fisher Exact Test (two-tail)			0.000

Table S3. Cohen's kappa measure of association for MICROSTRUCTURE_ASSIGN$ and SULFUR_ASSIGN$

Coefficient	Value	ASE	95 % Confidence Interval		Z	p-value
			Lower	Upper		
Cohen's kappa	0.616	0.106	0.409	0.823	5.835	0.000

Table S4. Millar's HISEA model results:

```
PROGRAM HISEA                        09-24-2008 at 16:19
 AFRP SPAWNING COMPOSITION ON MOKELUMNE RIVER 2004
 FUNCTION OF THIS RUN IS.........BOOTSTRAP
 #STOCKS IN THE MODEL............ 2
 THE STOCKS ARE..................HATCHERY WILD
 #VARIABLES USED................. 3

 STANDARD BEING RESAMPLED?.......Y
 RESAMPLED STANDARD SIZES........    50    50
 MIXTURE BEING RESAMPLED?........Y

 RANDOM NUMBER GENERATOR SEED....  123456
 NUMBER OF RUNS REQUESTED?.......  500

 THE SIZES OF THE STANDARDS ARE     64    82
 MIXED SAMPLE HAS    92 FISH
TABLE OF COMPOSITION ESTIMATE MEANS.  NUMBER OF RUNS =  500
```

	RAW	COOK & LORD	COOK CONSTRAINED	MILLAR CONSTRAINED	MAXIMUM LIKELIHOOD
HATCHERY	0.9269	1.0606	0.9924	0.9924	0.9708
WILD	0.0731	-0.0606	0.0076	0.0076	0.0292

TABLE OF COMPOSITION ESTIMATE STANDARD DEVIATIONS OVER THE 500 RUNS

	RAW	COOK & LORD	COOK CONSTRAINED	MILLAR CONSTRAINED	MAXIMUM LIKELIHOOD
HATCHERY	0.0308	0.0738	0.0202	0.0202	0.0342
WILD	0.0308	0.0738	0.0202	0.0202	0.0342

Table S5. Estimate of hatchery vs. natural origin in the 2004 adult CS escapement to the Mokelumne River watershed. This data analysis was performed by Michelle Workman of the East Bay Municipal Utility District (EBMUD).

	Number of Tagged Recoveries in 2004						
	4 year olds		**3 year olds**		**2 year olds**		
River	-	2	2	22	-	3	
Hatchery	-	12	2	432	2	244	
	Tags Recovered/proportion tagged						
River (estimate)	-	72	19	204	-	84	**Population Estimate based on expansion**
Hatchery (estimate)	-	255	15	3,184	56	8,935	12,823
	Proportion in the Population						
							By habitat
River	-	0.0056	0.0014	0.0159	-	0.0065	**0.03**
Hatchery	-	0.0199	0.0011	0.2483	0.0044	0.6968	**0.97**

Note: The 2004 escapement counts were 10,356 in the hatchery and 1,588 in the river.

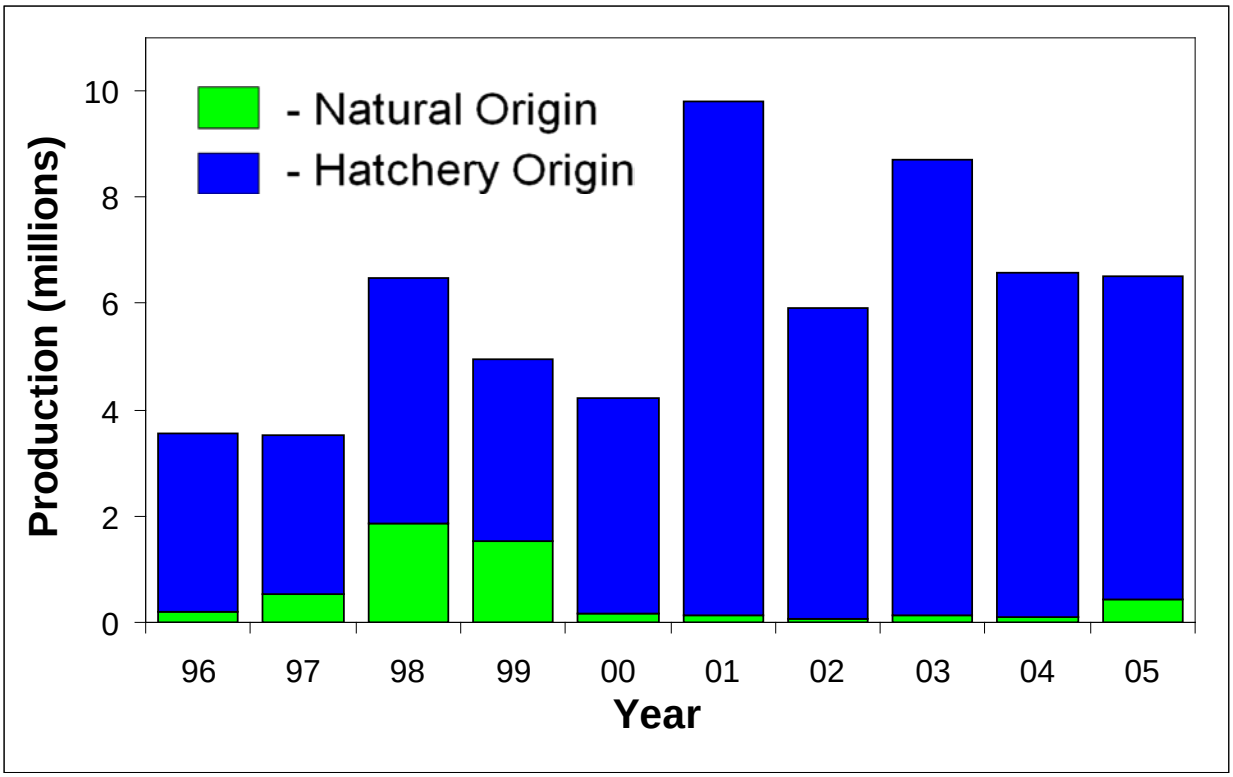

Fig. S1. Natural and hatchery production of juvenile CS in the Mokelumne River watershed.

Table S6. Projected cost of high volume microstructural and microchemical analysis with technician performing repetitive functions.

	Microstructure			**Microchemistry (sulfur isotopes)**		
			number of samples 500			number of samples 500
Task	otoliths per hour	Hours	Cost	otoliths per hour	Hours	Cost
whole oto photos	15	33	$ 666.67	15	33	$ 666.67
mount otos resin	20	25	$ 500.00	20	25	$ 500.00
polish 1 side	3	167	$ 3,333.33	3	167	$ 3,333.33
mount in rounds/ slide	30	17	$ 333.33	30	17	$ 333.33
polish 2nd side	1	500	$ 10,000.00	0.8	625	$ 12,500.00
gold coating				15	33	$ 666.67
ion probe imaging				10	50	$ 1,000.00
processing images (locating areas)				30	17	$ 333.33
increment width measurements	4	125	$ 2,500.00			
sulfur analysis				1	500	$ 31,000.00
post sample images				10	50	$ 1,000.00
data analysis		20	$ 400.00	12	42	$ 5,000.00
oversight (20% of total time)	2.8	177	$ 10,640.00	1.6	312	$ 28,050.00
instrument cost and supplies			$ 5,000.00	days of time	25	$ 37,746.67
travel and lodging				days for three	25	$ 12,666.67
Budget for time			$ 28,373.33			$ 84,383.33
Subtotal			**$ 33,373.33**			**$134,796.67**
Overhead			$ 5,840.33			$ 23,589.42
Total estimate for 500 otoliths			**$ 39,213.67**			**$158,386.08**
Estimated cost per otolith			$ 78.43			$ 316.77